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PROGRESS TOWARD DEVELOPMENT OF DIAGNOSTIC TESTS FOR GREEN TURTLE FIBROPAPILLOMATOSIS. PART II. IDENTIFYING ANTIGENS FOR DIAGNOSTIC TEST DEVELOPMENT EXPERIMENTAL TRANSMISSION OF GREEN TURTLE FIBROPAPILLOMATOSIS USING CELL-FREE TUMOR EXTRACTS

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ADMINISTRATIVE REPORT H-94-11C

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PROGRESS TOWARD DEVELOPMENT OF DIAGNOSTIC TESTS FOR GREEN TURTLE FIBROPAPILLOMATOSIS. PART II. IDENTIFYING ANTIGENS FOR DIAGNOSTIC TEST DEVELOPMENT: EXPERIMENTAL TRANSMISSION OF GREEN TURTLE FIBROPAPILLOMATOSIS USING CELL-FREE TUMOR EXTRACTS*

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PREFACE

This report was prepared in part as the result of a contract issued to Dr. Paul A. Klein by the Southwest Fisheries Science Center Honolulu Laboratory's Marine Turtle Research Program. The report describes significant progress recently made in research to experimentally transmit fibropapillomatosis using cell-free extracts in the green turtle, *Chelonia mydas*. This debilitating and often fatal tumor disease of unknown etiology represents a serious threat to green turtle populations in Hawaii, Florida, and certain other sites worldwide. The disease is believed to be increasing in both geographical scope and magnitude.

It is recommended that the reader also refer to Southwest Fisheries Science Center Administrative Report H-94-10C which constitutes Part I of the results of this contracted research by Dr. Klein.

Because this report was prepared by independent investigators, its statements, findings, conclusions, and recommendations do not necessarily reflect the views of the National Marine Fisheries Service, NOAA.

George H. Balazs
Zoologist
December 1994

ABSTRACT

Green Turtle Fibropapillomatosis (GTFP), characterized by multiple benign fibroepithelial tumors on the skin and eyes, has become a growing threat to green turtle (*Chelonia mydas*) populations worldwide. The cause of GTFP is unknown, but a viral etiology is suspected. This study investigated whether GTFP could be experimentally transmitted to young captive-reared green turtles using cell-free fibropapilloma extracts prepared from free-ranging turtles with spontaneous disease. Twenty green turtles were raised from eggs collected from 4 separate clutches in the wild. Five turtles from each clutch were assigned to one of 4 experimental transmission groups and 1 control (sentinel) group. For each independent transmission experiment a crude homogenate (33% w/v) was prepared from fibropapillomas removed from one of 4 free-ranging green turtles with spontaneous disease. The crude tumor homogenates were freeze-thawed and centrifuged to yield cell-free extracts that were used for inoculation, either without further processing or after being filtered (0.45 μm). Recipients were inoculated by intradermal injection or by scarification in selected anatomic sites. Sentinel turtles were not treated but were housed with treated turtles to control for spontaneous disease. Fibropapillomas developed at one or more inoculation sites in all 12 turtles receiving extracts from 3 of the four tumor donors. Both filtered and unfiltered tumor extracts successfully induced tumor development. During the 10 and 12 month monitoring periods, fibropapillomas did not develop in sentinel turtles, at untreated or sham (sterile saline) inoculated sites, or in any turtles inoculated with tumor extract from donor #4. The first fibropapillomas on individual turtles were detected between 15 and 43 weeks post inoculation. Although two sets of transmission experiments were performed 8 weeks apart, most of the tumors in both sets became evident simultaneously after water temperatures rose. Histologic examination revealed that the experimental tumors were identical to spontaneous fibropapillomas found in free-living turtles but lacked evidence of endo-parasites. Negative staining electron microscopy failed to reveal intact virus particles in transmission positive tumor extracts. This study has demonstrated that the etiology of GTFP is an infectious filterable subcellular agent. Once this agent is identified and isolated, experiments to fulfill Koch's postulates may be conducted. We will then be able to complete development of immunodiagnostic tests for monitoring green turtle populations for exposure to this disease.

KEY WORDS: Sea turtles · *Chelonia mydas* · Fibropapilloma · Disease transmission

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INTRODUCTION

Cutaneous papillomas, fibromas, and fibropapillomas were first described in green turtles, *Chelonia mydas,* over fifty years ago (Smith & Coates 1938, Lucké 1938, Smith & Coates 1939). The first case was from an adult green turtle at the New York Aquarium that had been captured near Key West, Florida and subsequent reports were from green turtles captured in Florida waters (Smith & Coates 1938, Lucké 1938, Schlumberger & Lucké 1948). Fibropapillomatosis has subsequently been observed in many geographic locations around the world including the western Atlantic and Gulf of Mexico (Jacobson et al. 1989, Ehrhart 1991, Teas 1991) the Caribbean (Meylan pers. comm., Jacobson et al. 1989, Teas 1991, Williams et al. 1994), the Pacific (Jacobson et al. 1989, MacDonald & Dutton 1990, Balazs 1991, Limpus & Miller 1994), and the Indian Ocean (Hendrikson 1958, J. Mortimer pers. comm.). In some well monitored sites, the prevalence of GTFP has increased markedly since the 1980's. For example, in Hawaii the first confirmed case of GTFP was from a juvenile green turtle captured in Kaneohe Bay, Oahu in 1958. A survey of local fishermen conducted by Balazs indicated that GTFP was rare to non-existent prior to this (Balazs 1991). Since 1989 the prevalence of fibropapillomatosis in sampling studies in Kaneohe Bay has ranged from 49 to 92% (Balazs 1991).

These documented increases in prevalence coupled with the fact that GTFP is often fatal in severely affected cases have raised concerns about the potential impact of GTFP on the long term stability of worldwide green turtle populations. These concerns have prompted efforts to identify the etiologic agent(s) and develop diagnostic reagents to monitor green turtle populations for exposure to this agent (Herbst in press).

The etiology of green turtle fibropapillomatosis is unknown. Histologically, fibropapillomas are characterized by benign papillary epidermal hyperplasia supported on broad stalks of proliferating fibrovascular stroma (Smith & Coates 1938, Lucké 1938, Smith & Coates 1939, Schlumberger & Lucké 1948, Jacobson et al. 1989, Harshbarger 1991, Aguirre et al. 1994, Williams et al. 1994). Agents known to cause similar proliferative cutaneous lesions in other species, including chemical carcinogens, ultraviolet light, oncogenic viruses, and metazoan parasites, have been proposed as possible causes of GTFP and have been reviewed by Sundberg (1991) and Herbst (in press).

The pattern of disease spread during GTFP outbreaks among captive green turtles is consistent with an infectious etiology (Jacobson 1981, Jacobson et al. 1989, Hoffman & Wells 1991). The presence of spirorchid trematode ova in the vasculature of many fibropapillomas (Smith & Coates 1939, Harshbarger 1984, Norton et al. 1990, Jacobson et al. 1991, Aguirre et al. 1994, M. Dailey pers. comm., E. Greiner pers. comm.) has lead some pathologists to suggest that GTFP represents a fibroplastic reaction to trematode ova (Harshbarger 1984). A herpesvirus has been identified in foci of epidermal ballooning degeneration in some fibropapillomas (Jacobson et al. 1991) however, this virus has not been isolated and Koch's postulates have not been fulfilled. This paper presents the findings of a controlled transmission study designed to test the hypothesis that GTFP is caused by a virus. The results indicate that fibropapillomatosis in green turtles is an infectious disease caused by a filterable subcellular agent rather than a metazoan parasite.

MATERIALS AND METHODS

Experimental animals. Green turtles to be used as recipients were obtained from the wild as eggs and raised in captivity. Five to seven eggs were collected from each of 4 green turtle nests that were deposited by different females within a one week period in August 1992 on Melbourne Beach, Brevard County, Florida. Three of the four nesting females were identified by flipper tags (S. Johnson pers. comm.). Following oviposition, each nest was marked and eggs were left in place until they were within 1-10 days of their expected hatching date (Rebel 1974). Eggs were then transported in separate plastic boxes filled with beach sand to a laboratory incubator (28.5°C and 95-100% relative humidity) where they remained through hatching, which occurred approximately 52 days post-oviposition (range 49-57 days). Newly hatched green turtles were marked according to clutch by cutting a small notch in one of the marginal scutes. Hatchlings were maintained in the lab in plastic tubs filled with filtered sea water and fed a commercial pelleted diet (Reptomin®, TetraWerke, Melle, Germany) for approximately two weeks. The babies were then transported by air to Marathon, Florida where they were housed by clutch in 4 large fiberglass tanks (useable capacity, 2400 l) on a continuous flow system providing approximately 20 volume changes per day of sand filtered (PACFAB TA60 sand filter) Florida Bay water. The turtles were fed floating trout chow (Purina, St. Louis, MO). The amount fed ranged from 1.5 to 1.75% body weight per day and was adjusted to prevent obesity, provide for moderate growth, and minimize cannibalism. The filters were back flushed daily. Tanks were vacuumed weekly and thoroughly cleaned monthly. Water temperatures, pH, and salinity (specific gravity) were monitored and recorded weekly. Twenty turtles (5 from each clutch) were raised until they were between 9 and 11 months old before experiments were begun.

Fibropapilloma donor turtles. Four free-ranging juvenile green turtles with multiple cutaneous fibropapillomas that stranded or were collected in Florida Bay or the lower Florida Keys were used as tumor donors in two sets of replicate transmission experiments carried out 8 weeks apart. Fibropapillomatosis was severe enough in these turtles to justify their removal from the wild to attempt rehabilitation. General anesthesia was induced in each donor turtle with a mixture of isoflurane (Aerrane®, Anaquest, Madison, WI) and nitrous oxide in oxygen delivered by mask and then by endotracheal tube. Once anesthetized, the turtles were draped and tumors were prepared by washing with copious amounts of sterile saline and scrubbing with a sterile brush. All cutaneous tumors were excised and incisions were closed with 2.0 nylon suture material (Dermalon®, American Cyanamid, Danbury, CT). Highly arborizing fibropapillomas with intact epithelium were selected for use in the transmission experiments. Ulcerated and necrotic masses were discarded. Each tumor was cut into quarters. Representative sections were taken from each quarter and either fixed in 10% buffered formalin or immersed in OCT medium (Tissue-Tek®, Miles Inc., Elkhart, IN) and frozen in liquid nitrogen. A portion of each mass was retained for tissue culture studies. The remaining pieces were placed in a sterile cup, weighed, and stored on dry ice until processing.

Preparation of cell-free tumor homogenates. Pooled fibropapilloma fragments from each donor were thawed and minced with a sterile scalpel and scissors and ground in a blender (Osterizer®, Sunbeam-Oster, Schaumburg, IL) in chilled sterile saline. The ground tumor was further homogenized in Ten Broeck glass tissue homogenizers (Pyrex, Corning, NY) on ice. This homogenate was frozen on dry ice and kept frozen for 15 minutes before being thawed and ground a second time. A second freeze - thaw cycle was then performed. Sufficient sterile saline was added during the homogenization process to yield a final 33% w/v crude homogenate. This crude homogenate was first centrifuged in a clinical centrifuge at 500 x g for 10 minutes to sediment large debris and the supernatant was collected. Centrifugation of the pellet was repeated once and the supernatant added to that previously collected. The supernatant was then centrifuged at 10,000 x g for 10 to 15 minutes in a Savant microcentrifuge to pellet cells and debris. Samples of this supernatant were examined microscopically to confirm the absence of intact cells. Approximately half of the cell-free supernatant was filtered through a 0.45 μm syringe tip filter (Acrodisc®, Gelman Sciences, Ann Arbor, MI) to remove contaminating bacteria and any remaining intact cells. The other half of the cell-free supernatant was used as an unfiltered extract. The final centrifugation pellets and aliquots of unfiltered and filtered supernatants were frozen in liquid nitrogen and stored at -150°C in a liquid nitrogen freezer.

Experimental treatments. Prior to treatment each recipient was manually restrained and a 1 ml blood sample was collected from the dorsal cervical sinus for plasma banking (Owens & Ruiz 1980). In each replicate experiment, tumor extract from one donor turtle was used to treat one recipient from each clutch. Treatments included intradermal injections using a 0.5 ml insulin syringe with a 27 gauge needle into the upper eyelid (0.1 ml), proximal margin of a large scale on the palmar surface of the front flipper (0.2 ml), proximal margin of a large scale on the dorsal surface of the rear flipper (0.2 ml), and instillation into scarified skin on the dorsum of the neck (0.1 ml) and shoulder (0.1 ml). Skin was scarified with an 18 gauge needle and the inoculum was worked into the tissue by multiple needle pricks with a 25 gauge needle and allowed to air dry for at least 15 minutes before turtles were returned to water. All treated turtles received inoculations of filtered tumor extract on the right side of the body. In addition half of the treated turtles received sterile saline (sham) inoculations and half received unfiltered extract inoculations at comparable sites on the left side of the body. On the neck, turtles received either unfiltered or filtered tumor extract. One turtle from each clutch was kept as a control (sentinel). Sentinel turtles received no inoculations but were maintained in the same tanks as their treated clutch-mates to control for waterborne or contact transmission by the putative infectious agent.

Treated turtles were held and observed for 12 months following inoculations. Turtles were visually inspected daily and palpated weekly for development of inflammation or masses. Observations were recorded weekly. Blood samples were collected at various times during the monitoring period.

Histopathology. Biopsies of normal skin and putative GTFP lesions were collected, using a 6mm biopsy punch or scalpel blade under 2% lidocaine local anesthesia, and fixed in neutral buffered 10% formalin for histopathologic examination.

Negative staining electron microscopy. Samples of 0.45 μm filtered tumor extracts that had successfully transmitted GTFP were examined for the presence of virus-like particles as follows. First, 10 μl samples of filtered tumor extract were applied to carbon coated 400 mesh copper grids and allowed to absorb for 10 to 30 seconds. The grids were drained of excess liquid with a filter paper wick and then immediately floated on a drop of 2% aqueous uranyl acetate for 30 seconds. The grids were drained of excess fixative and allowed to air dry before examination in the electron microscope. Second, 1 ml of filtered tumor extract was centrifuged at 12,000 x g for 20 minutes. The clarified supernatant was then centrifuged at 100,000 x g for 2 hrs in an Airfuge A-100/18 rotor (Beckman Instruments, Fullerton, CA). The pellet was resuspended in 40 μl distilled water and aliquots were adsorbed to carbon coated 400 mesh copper grids and prepared for electron microscopy as described above.

RESULTS

Table 1 summarizes information for each of the four donor turtles used in this study. The donor turtles were juveniles ranging from 35 to 59 cm straight carapace length. Each had multiple cutaneous fibropapillomas ranging in size from a few millimeters to over 9 cm in diameter involving the axillary and inguinal regions, front and rear flippers, neck, and eyelids. Two turtles, donor #2 (Everglades) and donor #4 (Coastie) were unable to maintain neutral buoyancy and floated high in the water. Both subsequently died and were found on necropsy to have multiple pale firm nodules (fibromas) in visceral organs including lungs, kidney, liver, and heart. The other two donor turtles were surgically treated and one has been released. Between 9.0 and 26.3 g of cutaneous fibropapillomas from each donor were used to prepare tumor extracts for use in transmission experiments.

Transmission experiments. Recipient turtles had grown to approximately 25 cm straight carapace length and were either 9 months old (groups #1 and #2) or 11 months old (groups #3 and #4) when used in these experiments. Flipper and tail biting became a problem as turtles grew and one of the control turtles died from wound infection before the experiments could be initiated. Biting was reduced by altering the feeding schedule from single to multiple feedings per day.

Four independent transmission experiments were conducted on the following dates: 6-7 July 1993 (groups #1 and #2 using homogenates from donors #1 and #2 respectively), and 3-4 September 1993 (groups #3 and #4 using homogenates from donors #3 and #4 respectively). Table 2 shows the results of inoculations with filtered tumor extracts after 10 and 12 months respectively. The three control (sentinel) turtles did not develop spontaneous tumors during this period. All twelve turtles inoculated with tumor extracts from donors 1, 2, and 3 developed tumors at one or more injection sites. The 4 turtles in group #4, that received tumor extract from donor #4, did not develop tumors. Tumors developed only at sites that were inoculated with GTFP extracts (Figure 1). Tumors did not develop at sham inoculated sites or at any uninoculated sites. Although several turtles incurred bite wounds on rear flippers and tails, fibropapillomas did not develop at these locations. Table 3 lists the frequencies of fibropapilloma development by the type of

inoculum and route of inoculation. Filtered GTFP extracts were no more successful than unfiltered extracts in inducing tumor development (58% of injection sites and 17% of scarification sites in 16 turtles versus 42% of injection sites and 19% scarification sites in 8 treated turtles). The differences in percent success between filtered and unfiltered extracts were not statistically significant (Chi-Square, $p > 0.10$). On the other hand, sites injected intradermally were more likely to develop tumors than scarification sites (Chi-Square, $p < 0.05$).

Time to tumor development. Figure 2 shows the time course of tumor development for individual turtles during this study. Earliest indications of tumor development were identified as slightly raised epidermal swellings ranging from 0.5 to 8 mm maximum diameter. The earliest tumors were detected at 15 weeks post inoculation in two turtles from replicate #3. The time lag between inoculation and observation of the first tumors to develop on individual turtles ranged from 14.6 to 43.4 weeks (mean = 26 $\pm$ 8.8 weeks). The average lag time for first tumor detection was 8 weeks longer for the 8 turtles in the first set of replicates (July) (mean = 28.6 $\pm$ 8.4) than for the 4 turtles in the second set (September) (mean = 20.6 $\pm$ 7.7 weeks), although this difference was not statistically significant (Mann-Whitney U test, 2-tailed, $0.05 < p < 0.10$). The average mean tumor detection time (averaged for all tumors on each individual) was 6.8 weeks longer for the 8 turtles in the first set (mean = 33.4 $\pm$ 5.2) than for those in the second set (mean = 26.6 $\pm$ 4.3 weeks) and this difference was statistically significant (Mann-Whitney U test, 2-tailed, $p < 0.05$). This difference roughly corresponds to the 8 week gap between sets of replicate experiments and suggests that tumors tended to appear synchronously. The majority of fibropapillomas (29 out of 36) were initially detected between early February and May regardless of when turtles were inoculated. Only 4 turtles had tumors develop within the first 4 months post inoculation. During the course of the experiment water temperatures ranged from 30°C in the summer to 17.5°C in the winter and were lowest (below 21°C) from December through January (Figure 2). Salinity and pH remained constant (1.027 sp. gr. and 8.2 respectively). The onset of colder water temperatures in December appeared to retard growth rates of early tumors. A similar effect on subclinical tumors could synchronize their appearance with the return to warmer water temperatures.

Histopathology. Thirty-five experimentally induced fibropapillomas from 11 recipient turtles were biopsied between 1 and 27 weeks following their detection. Lesions were raised, sessile or polypoid masses with verrucous or smooth surfaces and ranged from 0.5 to 2 cm in diameter when biopsied (see Figure 1). Tumors consisted of epidermal hyperplasia supported by arborizing proliferating fibrovascular stroma (Figure 3). Hyperplastic epidermis was between 7 and 15 cell layers thick. Most of the hyperplasia was in the stratum spinosum and cells in this layer were hypertrophic. There was extensive proliferation of fibroblasts within the papillary layer of the dermis. The dermis was hypercellular with fine collagen bundles arranged in an irregular pattern. Cells in the dermis were well differentiated spindle-shaped cells with a fine chromatin pattern. Perivascular mononuclear cell infiltrates were observed within the deeper layers of the dermis in most sections. No inclusions or trematode ova were observed within any sections. The histologic features of these induced lesions were consistent with spontaneous green turtle fibropapillomas (Jacobson et al., 1989).

Negative staining electron microscopy. No virus-like particles could be demonstrated in samples of transmission positive tumor extracts or in ultracentrifuge pellets prepared from 1 ml aliquots of these extracts by negative staining electron microscopy.

DISCUSSION

Fibropapillomas were induced in twelve healthy captive-reared juvenile green turtles using filtered cell-free extracts prepared from fibropapillomas collected from 3 out of 4 donors with spontaneous disease. These results provide the first experimental evidence that green turtle fibropapillomatosis is caused by an infectious agent under 0.45 μm in size, most likely a virus. The filtration step eliminated most bacteria (except mycoplasmas) and trematode ova as possible etiologies.

Several viruses have been associated with or shown to cause proliferative skin lesions in other vertebrate species. Papillomaviruses (papovaviridae) cause papillomas, fibromas, and fibropapillomas in many vertebrate species (Sundberg 1987) and have been observed in hyperplastic skin lesions of Bolivian side-necked turtles (Jacobson et al. 1982). In addition, papovavirus-like particles have been found in papillomas of green lizards (Raynaud & Adrian 1976, Cooper et al. 1982). A polyomavirus (papovaviridae) has been associated with cutaneous neoplasia include in hamsters (Graffi et al. 1968). Herpesviruses have been associated with cutaneous papillomas and/or fibromas in green lizards (Raynaud & Adrian 1976), African elephants, (Jacobson et al. 1986), carp (Sano et al. 1985, Hedrick et al. 1990), and several salmonid species (Kimura et al. 1981a, b, c, Sano et al. 1983, Yoshimizu 1987). Poxviruses cause proliferative skin lesions in squirrels (Hirth et al. 1969, O'Connor et al. 1980), rabbits (Shope 1932, Pulley & Shively 1973), and primates (Behbehani et al. 1968). Finally, certain retroviruses have been associated with fibromas and sarcomas in several species including walleyes (Martineau et al. 1991), angelfish (Francis-Floyd et al. 1993), cats (Hardy 1985), and non-human primates (Gardner & Marx 1985, Tsai et al. 1990).

Several surveys of GTFP biopsies for light and electron microscopic evidence of virus infection have been unsuccessful (Smith & Coates 1938, Jacobson et al. 1989, Aguirre et al. 1994). However, Jacobson et al. (1991) identified a herpesvirus in some fibropapillomas. Three fibropapillomas from one green turtle and 1 of 14 tumors examined from another had focal degenerative changes in the epidermis that contained eosinophilic intranuclear inclusions, confirmed by electron microscopy to be herpesvirus. This association of herpesvirus with GTFP may represent a secondary infection because herpesviruses have a tendency to colonize tumors and tissues of debilitated animals. We cannot conclude that this herpesvirus or any other virus is the etiologic agent of GTFP until it has been isolated and proven to be oncogenic in transmission experiments.

We hoped to identify virus particles in samples of transmission positive cell-free tumor extracts with negative staining electron microscopy. However, our initial studies have been unsuccessful. One explanation is that the agent is labile and was destroyed during prolonged storage or during thawing and sample preparation. Enveloped viruses

such as retroviruses and herpesviruses are more sensitive to storage and processing conditions. Although crude extracts were stored at -150°C we did not attempt to protect viruses from proteolytic enzymes. A second possibility is that the concentrations of intact viral particles within extracts were below the detection limits of the method. We suspect that this was the case because, for example, the herpesvirus discovered by Jacobson et al. (1991) was found in scattered foci within a small percentage of tumors. Similarly, papillomavirus vegetative replication occurs sporadically in only the most superficial terminally differentiated epidermal cells of a wart (Howley 1990). Successful detection by negative staining electron microscopy requires approximately 10^8 to 10^9 virus particles per ml (Doane 1992). A third possibility is that the agent that causes GTFP is present in tumors primarily as episomal genetic material which is infectious under experimental conditions. Several oncogenic viruses, including papillomaviruses (Howley 1983) and retroviruses (Benjamin & Vogt 1990) cause neoplastic lesions in tissues that are not permissive to vegetative viral replication. Thus, tumors develop and intact viral genomes are found within transformed cells but infectious virions are not produced. Episomal DNA of several papovaviruses, including hamster polyomavirus (Graffi et al. 1969) and cottontail rabbit papillomavirus (Ito & Evans 1961, Brandsma & Xiao 1993), have been shown to be infectious under experimental conditions. Finally, another but less probable explanation for our inability to detect virus particles in tumor extracts is that GTFP is caused by a sub-cellular infectious agent that is not detectable by electron microscopy such as a viroid (infectious nucleic acid) or prion (infectious protein) (Cohen et al. 1994).

This study was limited to 20 recipient animals because the green turtle in Florida is an endangered species. The experimental design was a 4 by 5 matrix that assigned recipient turtles from 4 separate clutches into 4 experimental groups and one control group. This design was chosen to minimize the chances of transmission failure due to donor factors such as the stage of tumor progression and the infectious dose of the putative agent, or recipient factors such as innate or acquired resistance and prior disease exposure. Turtles within a clutch were siblings or half-siblings and therefore more likely to share heritable disease susceptibility factors and to have had the same prior exposure to GTFP, e.g. vertical disease transmission from the mother. The fact that turtles from all 4 clutches developed GTFP suggests that all 4 clutches were susceptible to disease.

Transmission varied between fibropapilloma preparations however. The tumor extract prepared from donor #4 failed to induce fibropapillomas in any recipients. The infectious dose may have been too low in this extract or the tumors may have been at the wrong stage of development for infectious particle production. For example, bovine cutaneous fibropapillomas have a prepatent period during which productive papillomavirus infection cannot be detected (Olson et al. 1992). Exogenous factors may influence virus production and shedding in poikilothermic animals. For example, in Lucké (1938) renal adenocarcinoma of leopard frogs, which is caused by a herpesvirus, tumors are infectious only at the low temperatures required for the production and shedding of virus. Higher temperatures result in rapid growth and metastasis of non infectious tumors (McKinnell 1984).

The development of detectable tumors in experimental turtles showed a lag time ranging from 15 to 43 weeks. Although two sets of replicate experiments were conducted 8 weeks apart, most tumors developed concurrently. An exogenous factor such as season or water temperature may have helped to synchronize tumor development. Temperature effects on the development and growth rates of neoplasia have been well-documented in poikilotherms (Asashima et al. 1985, Bowser et al. 1990). While our data are not conclusive, future studies should examine the effects of different environmental temperatures on the efficiency of disease transmission and tumor development. The time lag for development of tumors caused by oncogenic viruses may also be influenced by infectious dose (Beard et al. 1955). Presently we have no way to estimate the dose of infectious agent in our tumor extracts.

The fact that trematode ova were not found within experimental lesions provides further evidence that trematode ova are not the cause of GTFP. In addition, experiments, in which suspensions of spirorchid trematode eggs were injected intradermally at several anatomic sites in three captive-reared yearling turtles, failed to induce tumors after more than one year of observation (Herbst et al. 1994). These findings coupled with the results of the present study rule out the direct involvement of trematode eggs in the pathogenesis of GTFP.

This study is the first to demonstrate the transmissibility of green turtle fibropapillomatosis. Future work will attempt to isolate virions and viral genomic sequences from transmission positive fibropapilloma extracts. If an agent can be identified and isolated from these extracts we will conduct further transmission experiments to try to fulfill Koch's postulates for this disease. Isolation of the infectious agent will be important in developing the diagnostic assays necessary for studying the epizootiology of this disease including monitoring green turtle populations for exposure, identifying reservoirs for the disease agent, and elucidating natural routes of transmission.

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Table 1. Free-ranging green turtles with cutaneous fibropapillomatosis used as fibropapilloma donors.

Identity	Capture / Stranding Date	Location	Straight Carapace Length (cm)	Wet weight of pooled fibropapillomas used in extract preparation (g)
Donor #1 "Flamingo"	28 June 1993	Florida Bay, Everglades National Park	35.6	9.0
Donor #2 "Everglades"	30 June 1993	Florida Bay, Everglades National Park	61.4	19.0
Donor #3 "Pappy"	25 August 1993	Florida Keys, Stock Island	58.5	26.3
Donor #4 "Coastie"	21 August 1993	Florida Keys, Bahia Honda Bridge	59.3	15.4

Table 2. Fibropapilloma development at inoculation sites in green turtles treate with filtered `cell-free fibropapilloma extracts.

Recipients Clutch	Experiment #1 (Donor #1)	Experiment #2 (Donor #2)	Experiment #3 (Donor #3)	Experiment #4 (Donor #4)	Control Group
A	+ (2/4)[a]	+ (2/5)	+ (3/5)	- (0/4)	-
B	+ (2/4)	- (2/5)	+ (4/4)	- (0/5)	N/A[b]
C	+ (1/5)	+ (2/4)	+ (3/5)	- (0/4)	-
D	+ (3/5)	+ (3/4)	+ (3/4)	- (0/5)	-

[a] "+" indicates tumor growth at one or more inoculation sites and "-" indicates no tumor growth at any treatment site. Data in parentheses are the number of anatomic sites where tumors developed over the number that were inoculated with 0.45 μm filtered cell-free tumor extracts. Sixteen turtles were inoculated by injection or scarification at 4 or 5 sites on the right side of the body with filtered extracts prepared from 4 donor turtles with spontaneous tumors. The control group was not inoculated. In addition, 6 of 8 turtles (2 each from experiments 1, 2, and 3) that were inoculated with unfiltered extract at comparable sites on the left side of the body developed tumors at one or more sites while the remaining 2 turtles from experiment 4 did not.

[b] N/A, this control turtle died before experiments were begun.

Table 3. Frequency of fibropapilloma development at injection and scarification sites in recipient green turtles.

Inoculum	Injection Sites[a]	Scarification Sites[b]
Unfiltered Tumor Extracts	10/24 (42 %)[c]	3/16 (19 %)
Filtered Tumor Extracts	28/48 (58 %)	4/24 (17 %)

[a] 0.1 - 0.2 ml of inoculum injected intradermally at 3 sites (upper eyelid, front, and rear flippers) on each turtle.

[b] 0.1 ml of inoculum applied to scarified skin of neck and shoulders.

[c] Data include turtles from experiment #4 in which tumors did not develop at any inoculation sites.

Figure 1. Experimentally induced cutaneous fibropapillomas in the green turtle. Representative tumors (arrows) were chosen to illustrate site specific development and gross appearance at the time of biopsy. Tumors developed at one or more inoculation sites in 12 of 16 recipient turtles from 3 of 4 independent transmission experiments. Recipient C-3 (upper left) with sessile, verrucous tumor on right upper eyelid induced by intradermal injection of filtered tumor extract. Recipient C-2 (upper right) sessile, smooth tumor on right front flipper induced by intradermal injection of filtered tumor extract. Recipient B-3 (lower left) sessile, verrucous tumor on right shoulder induced by scarification with filtered tumor extract. Recipient C-3 (lower right) pedunculated, verrucous tumor on right rear flipper induced by intradermal injection of filtered tumor extract.

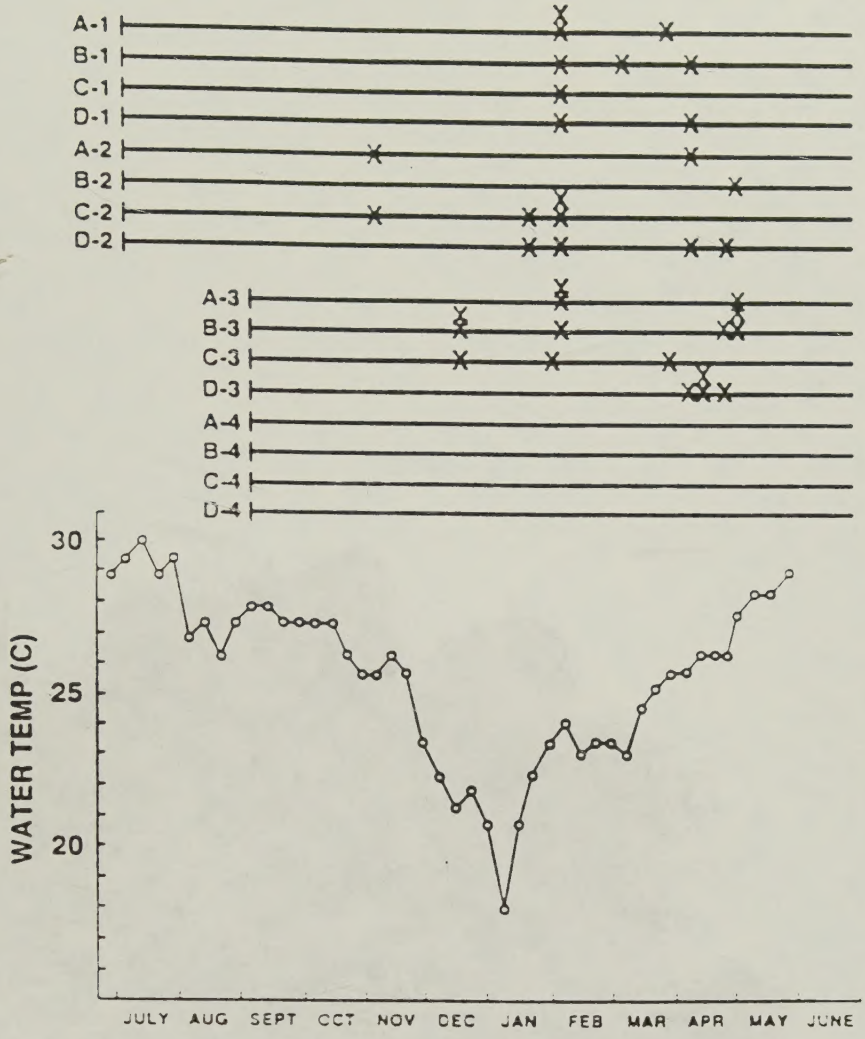

Figure 2. Time course for experimental fibropapilloma induction in the green turtle. Horizontal parallel lines above the XY graph represent the observation periods for each recipient turtle (A-1 to D-4) beginning with the dates of their inoculation with tumor extract. Dates on which tumors were first detected are indicated by X's. The XY graph indicates the fluctuation in water temperature (recorded weekly) during the course of this experiment.

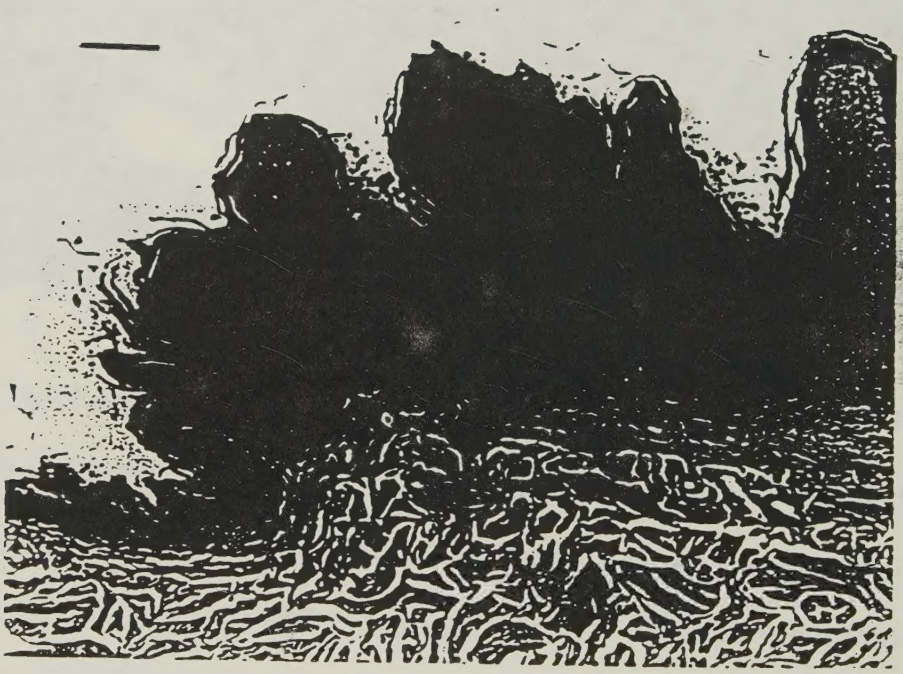

Figure 3. Experimentally induced fibropapilloma in the green turtle showing characteristic benign epidermal hyperplasia on broad fibrovascular stalks. These experimental lesions did not contain spirorchid trematode eggs. H&E (X 49, scale bar = 20 µm).